Secondary metabolite production by the fungal pathogen *Eutypa lata:* Analysis of extracts from grapevine cultures and detection of those metabolites *in planta*

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Abstract

Eutypa dieback of grapevines is caused by the fungal pathogen *Eutypa lata* and reduces vineyard longevity worldwide. Early detection could reduce losses due to this disease, so our aim was to identify acetylenic phenol metabolites of *E. lata* that could prove suitable as chemical markers in an early diagnostic test for the pathogen. Accordingly, secondary metabolite production by 30 isolates of *E. lata* grown on media derived from canes of three grapevine cultivars was analysed using HPLC. Six metabolites, namely eutypinol, methyl eutypinol, eulatachromene, eutypine, 2-*iso*-propenyl-5-formylbenzofuran and eulatinol, were detected in culture filtrates. Most abundant were eutypinol and methyl eutypinol, produced by 97 and 83% of isolates, respectively. There was no apparent correlation between secondary metabolite production on media containing milled canes from the three cultivars of grapevine, and the field tolerance of these same cultivars to Eutypa dieback. When various other fungi commonly isolated from grapevine trunks in Australia were grown on milled cane, no secondary metabolites characteristic of *E. lata* were detected, suggesting such compounds are specific to *E. lata*. To examine the detection of secondary metabolites *in planta*, micropropagated grapevine plantlets were treated with purified or crude culture filtrates from nine isolates of *E. lata* grown on malt yeast broth. Various secondary metabolites were identified in treated plantlets, however, no single compound was detected consistently. Eutypinol was detected in micropropagated grapevine plantlets inoculated with mycelium of *E. lata*, however, no metabolites were detected in the sap of vines which had been artificially inoculated with the pathogen.

Introduction

Eutypa dieback has a significant economic impact in vineyards worldwide. In some Australian vineyards more than 60% of vines show foliar symptoms of the disease (Wicks and Hall 1997), and up to 100% may exhibit the characteristic necrosis of woody tissue caused by *Eutypa lata* (Creaser and Wicks 2002). Studies in California showed that over 90% of vines may be affected by the time vineyards reach 20 years of age (Duthie et al. 1991), with older vineyards showing yield decreases of up to 83% (Munkvold et al. 1994). Ultimately, infected vines will die.

Shoot symptoms include dwarfing of internodes, leaf tattering and foliar necrosis. These symptoms are clearly visible in spring, although they quickly become obscured by vigorous growth from neighbouring healthy vines and may be confused with other diseases or disorders. There is considerable annual variation in symptom expression, with vines commonly displaying symptoms one year but not in the subsequent year (Creaser and Wicks 2001). Foliar symptoms have been attributed to toxins produced by *E. lata* in the vascular tissue of the wood. *E. lata* colonises vines through fresh wounds in mature wood and grows slowly throughout the vascular tissue. Foliar symptoms may not become evident until 3–8 years after infection (Carter 1991, Moller and Kasimatis 1978). Hence, by the time foliar symptoms are visible, the pathogen may have spread extensively throughout the vine.

DNA-based markers to identify *E. lata* within infected woody tissues are available (Lardner et al. 2005, Lecomté et al. 2000). However, this is a destructive assay. Furthermore, although DNA-based identification is a sensitive technique, this method relies on sampling host tissue that contains mycelium of *E. lata*, so that false negative results are possible if samples are taken from healthy tissue within an infected vine. In contrast, metabolites of *E. lata* are likely to be distributed throughout the vascular tissue and foliage of infected vines, especially in spring when

foliar symptoms are most evident. Hence, diagnosis based on identifying specific metabolites of *E. lata in planta* may enable early and more reliable detection of the pathogen before it spreads extensively throughout the vine.

The phytotoxin eutypine was originally implicated as the compound responsible for the foliar symptoms of Eutypa dieback (Tey Rulh et al. 1991). However, we previously found that eutypine is not produced by all isolates of *E. lata*, and that it readily undergoes facile cyclisation to a benzofuran compound (Molyneux et al. 2002). Furthermore, *E. lata* produced a range of other metabolites in artificial culture. Previous studies have shown that these metabolites have varying degrees of phytotoxicity, with some compounds, for example eutypinol and siccayne, showing no phytotoxicity when applied to grapevine leaf discs and others, including eutypine and eulatachromene, causing widespread necrosis of leaf discs (Mahoney et al. 2003, Smith et al. 2003). We also found that the amount and type of metabolite produced varied according to the fungal isolate and growth medium (Mahoney et al. 2003, Mahoney et al. 2005). Originally, we hoped that a chemical marker could be detected remote from the site of infection, in the sap or foliage of infected vines. We anticipated that low levels of metabolites of *E. lata* would be present before foliar symptoms became visible, and that this could be used as an early diagnostic tool for the pathogen. However, working *in vivo*, metabolites of *E. lata* have so far only been found within the woody tissues of infected vines.

Those metabolites could nevertheless prove diagnostic, and here we report on a series of experiments conducted to clarify what metabolites were consistently produced by *E. lata* in artificial culture and to obtain information on detecting these metabolites *in planta*. Because *E. lata* is an out-crossing species that displays considerable genetic and pathogenic variation (Péros and Berger 2003, Sosnowski et al. 2006), we analysed 30 isolates to obtain a representative picture of secondary metabolite production by the pathogen.

Firstly, we analysed secondary metabolite production by 30 isolates of *E. lata* which were grown on milled cane from three cultivars of grapevine. Our goals were (1) to identify a chemical marker that was produced by the majority of isolates of *E. lata* and (2) to determine whether cultivar tolerance to Eutypa dieback influenced the amount or type of secondary metabolite produced. We also analysed secondary metabolite production by 18 isolates of other grapevine-inhabiting fungi after growth on milled grapevine cane to confirm that the metabolites detected here were specific to *E. lata*.

Secondly, we grew nine isolates of *E. lata* on malt yeast broth, previously shown to be conducive to the production of a range of metabolites by *E. lata*. Micropropagated grapevine plantlets were then treated with purified or crude filtrates taken from these cultures. Our aim was to see whether metabolites of *E. lata* could be detected in grapevine tissue, and to gather information about the fate of such metabolites *in planta*.

Thirdly, to determine whether chemical markers indicative of *E. lata* could be detected in infected vines, we used HPLC to identify such metabolites in both micropropagated and potted vines which had been previously inoculated with mycelium from *E. lata*.

Methods

Isolates and culturing

Isolates of *E. lata* (Table 1) were obtained from perithecia on mature wood or from hyphae in infected cordons, as described by Carter (1991). Cultures were maintained in the dark on potato dextrose agar (PDA, Difco) at 25°C and stored in sterile distilled water at 4°C. For secondary metabolite analysis, media were derived from 1-year-old dormant canes of cultivars Merlot, Semillon and Shiraz, which were ground in a Wiley mill with a 1 mm screen. Each isolate was cultured in a 250 mL flask containing 10 g ground cane, 1 g sucrose and 50 mL reverse osmosis water that was sterilised by autoclaving. Flasks were inoculated with 5–8 mycelium plugs (approximately 2 square millimetres), taken from the margin of actively growing colonies on PDA. Cultures were maintained in the dark at 25°C for 20 d. Species other than *E. lata* were cultured on Shiraz medium only. These species included five isolates of *Phaeomoniella chlamydospora*, three isolates each of *Botryosphaeria* sp. and *Fomitiporia* sp., two isolates each of *Cryptovalsa ampelina* and *Phaeoacremonium aleophilum* and one isolate each of *Fomitiporia australiensis*, *Botryosphaeria ribis* and *Libertella* sp.

Isolation and analysis of secondary metabolites specific to E. lata

After incubation for 20 d, cultures were mixed with 100 mL double distilled (dd) water and mycelium was disrupted using a spatula. The resulting suspension was sonicated for 15–30 s then filtered through Whatman No. 4 filter paper. The filtrate was partitioned with an equal volume of chloroform and the aqueous phase discarded. The organic phase was partitioned with an equal volume of water, the aqueous phase discarded and chloroform was removed under reduced pressure. The residue was re-suspended in 10 mL acetonitrile, and liquid removed under reduced pressure. The residue was dissolved in methanol (1 mL) and filtered through a 0.45 µm 3 mm syringe filter (Gelman). Samples (20 µL) were analysed by HPLC using a Microsorb C_{18} 5 µm column, 250 × 4.6 mm i.d. (Varian) with a gradient elution at 1 mL/min of 100% water with 0.5% acetic acid to 100% acetonitrile over 30 min and held at 100% acetonitrile for 5 min. Photodiodearray detection (Agilent 1100 DAD) was used to identify metabolites. Amounts of previously characterised compounds were determined by reference to standard curves prepared for each compound. Reference standards were synthesised as described by Smith et al. (2003) and were linear over the range tested, 0.2–20 µg/20 µL injection. Amounts of any previously unidentified metabolites of *E. lata* were not quantified, but were instead represented by either '+' for presence or '–' for absence.

Detection of secondary metabolites in micropropagated vines treated with metabolites of E. lata

To see whether secondary metabolites of *E. lata* could be detected *in planta*, micropropagated grapevine shoots and

Table 1. Summary of secondary metabolite production by 30 isolates of *E. lata* on milled cane from three grapevine cultivars.

Isolate	Host species & origin	Metabolites (µg)														
		Eutypinol			Methyl eutypinol			Eulatachromene			Eutypine			Benzofuran[1]		
		Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon
01017B	*Vitis vinifera*, NSW	63	19	13	3.3	1	1.2	—	—	—	1.1	—	—	—	0.4	0.1
1776	*V. vinifera*, SA	2.5	—	1.5	—	—	—	—	—	—	—	—	—	—	—	—
200/2	*V. vinifera*, SA	351	440	586	23	5.2	13	26	28	21	2.9	2.4	—	3	—	0.9
200/3	*V. vinifera*, SA	466	111	615	43	9.4	31	25	—	23	16	—	19	1	6.1	1.1
83330	*Prunus persica*, SA	1.1	1.8	96	—	—	8.8	—	—	10	—	—	2.6	—	—	1.1
83339	*Quercus* sp.	661	236	1380	126	7.4	138	28	5.5	36	—	—	—	—	0.5	—
Bx 1-10	*V. vinifera*, France	197	35	282	59	2	24	—	—	4.9	—	—	5.1	3.5	2.4	1.5
E1	*V. vinifera*, SA	295	56	81	47	5.2	3.8	—	—	—	12	—	0.7	10	0.7	0.6
E120	*V. vinifera*, California	172	88	180	—	—	—	5.3	5.3	8.8	4.2	0.7	13	0.3	1.9	3
E125	*V. vinifera*, California	869	255	547	47	1.7	14	24	11	17	57	1.8	—	75	1.9	16
EL3	*V. vinifera*, S. Africa	387	266	130	53	11	15	28	11	3.8	—	1.5	—	1	0.6	0.4
EL4	*V. vinifera*, S. Africa	114	279	103	28	10	4.9	12	21	5.6	27	9.1	1.1	1.2	3.1	0.9
HT01	*Prunus armeniaca*, SA	491	105	417	59	4.8	15	20	1.5	9.3	7.6	2	4.1	2.1	1.4	1.3
JB16	*V. vinifera*, SA	706	486	701	164	43	84	33	16	47	—	19	—	—	12	—
M266	*V. vinifera*, France	1070	13	137	—	—	1.3	—	—	1.3	18	—	—	—	0.1	1.9
M279	*Prunus armeniaca*, Vic	—	7.8	20	7.0	6.2	27	—	1	—	—	—	7.3	—	2.7	27
M280	*V. vinifera*, Vic	179	522	491	11	8.5	—	0.8	6.9	7.3	2.7	4	18	12	11	—
M295	*V. vinifera*, Vic	—	—	1.4	—	—	—	—	—	—	—	—	—	—	—	—
M302	*V. vinifera*, Vic	131	41	75	9.6	1.5	2.6	4.2	—	1.8	12	—	1.2	1.2	0.4	0.1
M335	*V. vinifera*, Vic	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
NO3	*V. vinifera*, SA	303	19	82	51	0.9	3.9	—	—	—	—	—	—	10	0.4	4.2
NO4	*V. vinifera*, SA	610	199	487	96	21	55	13	4.5	11	18	—	—	6	3.6	12
NO7	*V. vinifera*, SA	216	224	310	259	36	85	9.7	8.7	7.8	—	—	—	10	0.3	1
NO8	*V. vinifera*, SA	421	39	376	23	1.6	9.2	12	—	8	9.4	—	—	2.1	0.5	9
P#1 SS12	*Pyrus communis*, SA	—	25	19	—	—	—	—	—	—	—	—	—	—	0.4	0.1
RB440	*V. vinifera*, France	24	203	126	7.2	18	21	—	4.7	3.5	7.2	4.2	12	2	2.2	3.6
SS2	*V. vinifera*, SA	—	—	48	—	—	4.5	—	—	1.6	—	10	1.3	—	—	37
SS6	*Viburnum opulus*, SA	3.8	16	18	6.1	2.3	7.3	—	—	—	1.4	1	3.1	2.7	6.3	9
SS10	*V. vinifera*, SA	3.8	44	17	0.3	2	1	—	—	—	0.05	—	—	—	0.3	0.1
SS11	*V. vinifera*, SA	2	12	9.3	0.5	1.3	1.2	—	—	—	—	0.2	0.3	0.2	—	—
	Total	7738	3745	7350	1124	199	572	241	125	229	196	56	89	144	59	132

— = not detected

[1] 2-*iso*-propenyl-5-formylbenzofuran

Detection limits (µg/mL) at 254 nm:

Eutypinol	0.03	Benzofuran	0.005
Methyl eutypinol	0.03	Eulatinol	0.01
Eulatachromene	0.05	Siccayne	0.01
Eutypine	0.01		

plantlets (cultivar Cabernet Sauvignon) were treated with purified or crude culture filtrates from nine isolates of *E. lata* (isolates E1, E120, E125, M266, M279, M280, M335, NO4 and SS6) grown on malt yeast broth (MYB; 10 g/L malt extract, 20 g/L yeast extract, Difco). MYB was selected for use in this experiment as we have previously shown that *E. lata* produces a wide range of secondary metabolites on this medium (Mahoney et al. 2003).

Conical flasks (250 mL) containing 50 mL MYB were inoculated with *E. lata* as above. Cultures were maintained for 30 d at 25°C in the dark. Three cultures were initiated for each isolate. After 30 d, replicate cultures were pooled, then sonicated (15–30 s) and filtered through Whatman No. 4 filter paper. The filtrate was divided into three equal portions. Two portions were extracted to provide purified filtrates, as described above, except that di-ethyl ether was used as the solvent rather than chloroform. The first purified filtrate was re-suspended in methanol and analysed using HPLC as described above. This provided a reference sample of known composition for each of the nine isolates.

The second purified filtrate from each isolate was re-suspended in ethylene glycol (200 µL) then added to 5 mL sterile dd water. Shoots (four internodes and the apex) were excised from micropropagated grapevine plantlets, placed in the resulting 5.2 mL of purified filtrate and incubated at 25°C (16 h light, 8 h dark) in 120 mL polypropylene tissue culture vessels for 10 d. After 10 d, shoots were rinsed in dd water to remove any filtrate, and dried on paper towel. Shoots were then ground in liquid nitrogen, mixed in 20 mL dd water, sonicated for 60 s and filtered through Whatman No. 4 filter paper. The filtrate was partitioned with an equal volume of di-ethyl ether, and

metabolites extracted and analysed by HPLC as described above. Following the removal of excised shoots, metabolites were also extracted from the ethylene glycol/water solution by making the volume up to 20 mL with dd water, filtering through Whatman No. 4 filter paper, then extracting with di-ethyl ether and subjecting to HPLC as described above.

The third portion of each MYB culture was sterilised by passing through a 0.2 µm filter, and the resulting crude filtrate (50 mL) was applied to the roots of micropropagated grapevine plantlets established on filter paper bridges in half-strength liquid MS medium (Murashige and Skoog 1962) in 250 mL polypropylene tissue culture vessels. The MS medium was removed before the addition of the crude filtrate. Plantlets were exposed to crude filtrates for 10 d, then cut off at root level and rinsed in dd water. Shoots were ground in liquid nitrogen, sonicated and extracted with di-ethyl ether as described above. Metabolites were also extracted from the remaining material (a mixture of plantlet roots and crude filtrate) by grinding in liquid nitrogen and extracting with an equal volume of di-ethyl ether. The extracts were then analysed by HPLC as above. Control plants, treated with solvent or MYB only, were included for each treatment.

Detection of secondary metabolites in micropropagated vines inoculated with mycelium of E. lata

Micropropagated grapevine plantlets (cultivar Cabernet Sauvignon) were inoculated with mycelium from five of the nine isolates of *E. lata* used above (isolates E1, E120, E125, M279 and M280). Six plantlets were inoculated for each isolate, and six uninoculated control plantlets were included. Plantlets were maintained on half-strength MS medium at 25°C (16 h light, 8 h dark) and were inoculated by placing slivers of mycelium taken from the margins of actively growing cultures on fresh wounds made on the stem of plantlets. Mycelium was held in place by applying a small amount of molten water agar immediately after inoculation. Plantlets were assessed for foliar symptoms 42 days after inoculation and metabolites were extracted and analysed using HPLC as described above.

Detection of secondary metabolites in infected vines

Metabolites were also extracted from the sap of potted vines which had been artificially inoculated with mycelium of ten isolates, individually, of *E. lata* in the shadehouse (Sosnowski et al. 2005a). For each isolate, two vines were selected, one which displayed intense foliar symptoms of Eutypa dieback within 8 months of inoculation, and one which had been inoculated with the same isolate but was asymptomatic. Sap was extracted from each vine 20 months after inoculation using a pressure chamber. This was achieved by placing the entire vine in the chamber, which was pressurised until sap began exuding from a freshly cut petiole. A pressure chamber was used on the assumption that metabolites of *E. lata* are present in the xylem sap of infected vines, which is exuded when vines are placed under pressure. For each vine, sap was collected over a four-hour period. Sap was also extracted from ten control vines, five of which were inoculated with blank agar plugs and five of which were uninoculated. Metabolites were extracted from the sap using di-ethyl ether and analysed using HPLC as described above.

Results

Isolation and analysis of secondary metabolites specific to E. lata

Five phenolic metabolites, namely eutypinol, methyl eutypinol, eulatachromene, eutypine and its benzofuran derivative, 2-*iso*-propenyl-5-formylbenzofuran, were commonly detected in extracts from cultures of *E. lata* grown for 20 d on milled wood of each cultivar. A quinol compound, eulatinol, was also produced by some isolates. Secondary metabolite production by all isolates on each cultivar is summarised in Table 1. Four of the 30 isolates produced all six metabolites, 14 isolates produced five metabolites and seven produced four metabolites. One isolate, M335, produced no secondary metabolites that were detectable by HPLC; the remaining isolates of *E. lata* produced between one and three metabolites. The other species tested did not produce any detectable acetylenic phenol metabolites.

The most frequently produced and abundant compound was eutypinol, produced by 29 of the 30 isolates (all except M335). Each of the 29 isolates produced eutypinol when grown on Semillon. Three isolates (1776, M295 and SS2) did not produce eutypinol on Merlot, and four (M279, M295, P#1SS12 and SS2) did not produce eutypinol on Shiraz. Amounts of eutypinol varied depending on fungal isolate and cultivar. For example, isolate M266 produced 1070, 137 and 13 µg of eutypinol following growth on Shiraz, Semillon and Merlot, respectively. However, there was no correlation between amount of eutypinol and cultivar; whereas isolate M266 produced most eutypinol on Shiraz, isolates 83339 and RB440 produced maximum amounts on Semillon and Merlot, respectively (Table 1). The amount of eutypinol produced ranged from 1 µg (isolate 83330, Shiraz) to 1380 µg (83339, Semillon). Eutypinol comprised 82% of the total metabolites produced when isolates were grown on Shiraz, 88% on Semillon and 90% on Merlot. Methyl eutypinol was also relatively abundant, produced by 25 isolates in amounts ranging from 0.3 µg (SS6, Shiraz) to 259 µg (N07, Shiraz). Taken together, eutypinol and methyl eutypinol comprised approximately 94% of the total acetylenic phenol metabolites produced on each cultivar.

Eutypine, present in smaller quantities than eutypinol or methyl eutypinol, was produced by 23 isolates, in amounts ranging from 0.05 µg (SS10, Shiraz) to 57 µg (E125, Shiraz). Six isolates produced eutypine on milled wood of all three cultivars, seven on two cultivars and ten isolates on a single cultivar. The benzofuran compound, eulatachromene and eulatinol were produced by 27, 20 and 5 isolates, respectively (Table 1).

There was no apparent correlation between cultivar and type of secondary metabolites produced. On each cultivar, eutypinol was predominant, followed by methyl eutypinol and eulatachromene. The benzofuran compound was the next most predominant metabolite on Semillon and Merlot, whereas on Shiraz slightly more

eutypine was produced than benzofuran. Eulatinol, not detected in extracts from isolates grown on Merlot, was produced in the smallest amounts, by five isolates. Total metabolite production was considerably greater on Shiraz and Semillon (9457 and 8379 µg, respectively) than on Merlot (4184 µg).

Detection of secondary metabolites in micropropagated vines treated with metabolites of E. lata

Following growth of nine isolates of *E. lata* on MYB, the six compounds identified above, and two other compounds, methyl eutypine and siccayne (Deswarte et al. 1996, Molyneux et al. 2002), were detected in purified culture filtrates analysed by HPLC. Eutypinol was the predominant compound, but was not produced by every isolate. Control shoots treated with ethylene glycol did not display any symptoms following 10 d of exposure. Excised shoots treated with metabolites from isolates E1 and M266 displayed bleaching of stems and necrosis of foliage after 10 d; shoots treated with metabolites from the remaining isolates were asymptomatic. All plantlets treated with crude MYB filtrate displayed widespread necrosis; this may be due to the pH of the medium, which becomes alkaline following incubation of *E. lata* (N. Mahoney, unpublished data).

Detection of metabolites of *E. lata* in treated plant material was somewhat inconsistent, with yields from treated plant material generally small relative to their amount in the purified culture filtrate. Analysis of excised shoots treated with purified culture filtrates showed that eutypinol could be detected in these shoots, although always in small quantities, and only when shoots were treated with extracts from isolates that produced more than 100 µg of eutypinol as detected in the purified culture filtrate analysed by HPLC. Other compounds, including methyl eutypinol, eulatachromene, benzofuran and eulatinol were also detected in excised shoots exposed to purified culture filtrates. Although eutypine was present in the filtrates of some isolates, it was never detected in treated plant material. The amount of metabolites recovered was generally less in rooted plantlets treated with crude filtrates than in excised shoots treated with purified filtrates. Metabolites detected in plant material treated with filtrates from representative isolates are shown in Table 2.

Two novel compounds, with retention times of 16.5 and 19.5 min, were also detected. These compounds, which have not been fully characterised, were commonly present at higher levels in treated shoots and plantlets than in the purified culture filtrate analysed by HPLC; the compound with a retention time of 19.5 min was not present in purified culture filtrates, but was detected in excised shoots treated with purified filtrates and in plantlets treated with crude filtrates, as well as in the reservoir of ethylene glycol/filtrate solutions remaining after exposure to excised shoots (Table 2).

Detection of secondary metabolites in micropropagated vines inoculated with mycelium of E. lata

Forty-two days after inoculation, some, but not all plantlets displayed foliar symptoms similar to those seen in vines naturally infected with *E. lata*. HPLC analysis identified eutypinol in plantlets inoculated with mycelium from four isolates (all except E125), however, no other metabolites characteristic of *E. lata* were detected. Eutypinol was detected in symptomatic and asymptomatic plantlets, but never in more than two out of six replicates.

Detection of secondary metabolites in infected vines

HPLC analysis of sap taken from potted vines which had been inoculated with *E. lata* did not identify any characteristic metabolites in symptomatic or asymptomatic vines. The volume of sap exuded from each vine over a four-hour period varied from approximately 1 to 26 mL and was generally considerably less in infected vines, presumably because the xylem tissue had become blocked following infection by *E. lata*.

Discussion

Our present study provides the first report of analysis of secondary metabolites from a wide range of isolates of *E. lata* grown on milled cane from various grapevine cultivars. The study supports the suggestion by Mahoney et al. (2003) that eutypinol is suited for use in an early diagnostic test based on secondary metabolite production *in vitro*. However, methyl eutypinol and the benzofuran compound, which were produced by 83 and 90% of isolates respectively, may also be suitable chemical markers.

Eutypinol is not phytotoxic when applied to grapevine leaf discs (Mahoney et al. 2003, Smith et al. 2003). However, this absence of phytotoxicity does not preclude using eutypinol as a chemical marker. Rather, pathogenicity of *E. lata* is not solely a consequence of the production of phenolic metabolites, but reflects the ability of the pathogen to colonise grapevine wood and degrade the xylem tissue. Hence, the presence of *E. lata* in vines as illustrated by a non-toxic metabolite such as eutypinol is sufficient indication of infection. Based on clinical evidence, the pathogen will eventually colonise the entire trunk or cordon, either killing the entire vine or a significant portion of it, regardless of whether foliar symptoms are expressed.

Eutypinol was produced by 29 isolates, however not every isolate produced eutypinol on every cultivar. Similar patterns of production were seen for other compounds. This is consistent with studies showing that production of certain metabolites by *Fusarium* spp. appears to be species-, isolate- and substrate-specific (Doohan et al. 2003). Factors that influence fungal secondary metabolite production remain unclear (Betina 1989, Kokkonen et al. 2005), with different compounds commonly produced on different substrates. Indeed, for *E. lata*, eulatinol was produced almost exclusively on artificial media, in particular on MYB, whereas other compounds, for example eutypine and eulatachromene, were more abundant when cultured on grapevine extracts (Mahoney et al. 2003).

Incubating the pathogen on cane extracts rather than artificial media was expected to provide a more accurate indication of metabolite production *in planta*. However, this technique would not provide information on plant-

Table 2. Detection of secondary metabolites in plantlets exposed to purified and crude culture filtrates from representative isolates of *E. lata* for 10 days

Isolate	Treatment	Metabolites (µg)									
		Eutypinol	Methyl eutypinol	Eulata-chromene	Eutypine	Methyl eutypine	Benzo-furan	Eulatinol	Siccayne	rt 16.5 min[1]	rt 19.5 min[1]
E125	Purified filtrate (HPLC)	203	4.1	7.8	6.1	3.5	10	21	—	+	-
	Excised shoot treated with purified filtrate	0.8	0.7	—	—	—	—	—	—	+	+
	Ethylene glycol solution	93	22	2.7	—	—	—	—	—	+	+
	Rooted plantlet treated with crude filtrate	—	—	—	—	—	—	—	—	+	-
	Crude filtrate/roots	3.7	12	—	—	—	—	35	—	+	+
M266	Purified filtrate (HPLC)	1273	—	58	21	—	3.6	—	—	-	-
	Excised shoot treated with purified filtrate	10	0.3	5.9	—	—	0.4	—	—	-	-
	Ethylene glycol solution	409	—	31	13	—	2.8	—	—	+	-
	Rooted plantlet treated with crude filtrate	0.2	—	—	—	—	—	—	—	+	-
	Crude filtrate/roots	36	—	—	12	—	5.6	—	—	+	+
N04	Purified filtrate (HPLC)	269	149	7	—	1.6	2.7	78	—	+	-
	Excised shoot treated with purified filtrate	2.4	2.7	—	—	—	0.1	1.3	—	-	+
	Ethylene glycol solution	148	81	6.3	—	1	5.5	45	—	+	-
	Rooted plantlet treated with crude filtrate	0.5	0.6	—	—	—	0.3	1.1	—	+	+
	Crude filtrate/roots	26	18	—	19	1.6	3.6	59	—	+	+

— = not detected

[1] Quantitative data for metabolites with retention times of 16.5 and 19.5 min could not be determined as these compounds have not been fully characterised. Presence of these compounds is therefore indicated by '+', and absence by '-'.

fungal interactions. For example, enzymatic detoxification of eutypine to eutypinol in living grapevine tissue, as reported by Colrat et al. (1999), would not occur on autoclaved cane. Likewise, the age of the cane may influence the metabolism of the pathogen – an outcome previously reported by Ferreira (1999) and Schmidt et al. (1999), who observed differences in growth rates and production of extracellular hydrolytic enzymes when growing *E. lata* on annual cane versus mature wood, respectively. Hence, the age of the wood on which *E. lata* is grown may also influence production of acetylenic phenol secondary metabolites.

Two factors known to influence secondary metabolite production are temperature and water status (Betina 1989, Kokkonen et al. 2005); this has been illustrated for various fungi, including *Phoma* sp. and *Fusarium* sp. (Baxter et al. 1998, Doohan et al. 2003). These factors have not been examined in relation to secondary metabolite production by *E. lata*. However, recent research (Sosnowski et al. 2005b) showed that high rainfall in October (spring) may be related to reduced severity of foliar symptoms of Eutypa dieback in some South Australian vineyards. Conversely, extremely low rainfall in spring may also be related to a decrease in the severity of foliar symptoms (Sosnowski et al. 2005b). It is possible that certain toxic secondary metabolites of *E. lata* are synthesised only in particular conditions. If metabolite production is strongly influenced by temperature, water availability or other environmental factors, this may help explain the variation in severity of foliar symptoms of Eutypa dieback observed from year to year (Creaser and Wicks 2001). Vineyard management practices, such as regulated deficit irrigation (Goodwin and Jerie 1992), could also influence the expression of foliar symptoms. Further research is clearly required to elucidate the complex interactions between the host, pathogen and environment on fungal establishment on host vines and progress of this disease.

Isolate M335 was confirmed as *E. lata* using DNA markers specific to the pathogen (Lardner et al. 2005). However, this isolate did not produce any secondary metabolites detectable by HPLC. Santos et al. (2002) reported inconsistent production of certain secondary metabolites following storage of isolates of *Penicillium expansum*. Conceivably, the response of fungi to preservation may be isolate-specific (Ryan et al. 2001, Santos et al. 2002), so it is possible that isolate M335, obtained from perithecia on dead grapevine wood, may have lost the ability to produce secondary metabolites following storage.

In this study, two cultivars that are tolerant to the effects of Eutypa dieback (Merlot and Semillon) and one susceptible cultivar (Shiraz) were selected to assess the influence of cultivar on secondary metabolite production. Although the metabolite profiles of individual isolates varied considerably, the general pattern of metabolite production was similar among cultivars, with eutypinol predominant. The total amount of secondary metabolites produced on Merlot was less than half that on Shiraz, however, a similar total amount of metabolites was produced on Semillon and Shiraz. In this respect then, there was no apparent correlation between amount or type of secondary metabolites produced, and tolerance of the grapevine cultivar to Eutypa dieback.

Analysis of micropropagated plantlets treated with cul-

ture filtrates showed low yields of metabolites from plant material. Although eutypinol was detected in excised shoots treated with purified culture filtrates, it was detected less consistently in intact plantlets treated with crude culture filtrates. This may be due to poor uptake of compounds by rooted plantlets, however, other compounds, in particular eulatinol, could be detected in rooted plantlets as well as in excised shoots. This implies that failure to detect some compounds is more likely caused by other factors, such as degradation of compounds when in aqueous solution, or perhaps by *in planta* conversion to compounds not detected using HPLC.

The detection of previously unidentified metabolites in treated plantlets, which were absent or present in very small amounts in purified culture filtrates, provides some evidence for the transformation of metabolites *in planta*. Time course experiments, in which metabolites are extracted from plantlets treated with identical culture filtrates after various periods of exposure, may provide more information on detecting these compounds *in planta*. Likewise, exposing plantlets to individual metabolites would provide information on the uptake and possible degradation of these compounds. Such transformed metabolites may prove to be suited for use in a rapid diagnostic test if they can be shown to be a reliable indicator of infection in diseased vines. Because other compounds such as methyl eutypinol and the benzofuran compound could be detected in treated plantlets, even when present in smaller amounts than eutypinol in the purified culture filtrates analysed by HPLC, the possibility of using these compounds as chemical markers may warrant further investigation.

HPLC analysis of plantlets inoculated with mycelium of *E. lata* showed that eutypinol could be detected in plant material infected with the pathogen. The detection of eutypinol in asymptomatic plantlets supported the hypothesis that metabolites of *E. lata* may be present in infected vines before foliar symptoms become visible. However, neither eutypinol nor any other metabolites were detected consistently in symptomatic plantlets inoculated with the pathogen. Perhaps the non-lignified tissue of micropropagated plantlets is not an appropriate substrate for the production of secondary metabolites by *E. lata*.

Our inability to detect any metabolites in the sap of symptomatic vines inoculated with characterised isolates of *E. lata* implies that these metabolites may not be suitable chemical markers for diagnosing Eutypa dieback. The observation by Mahoney et al. (2005) that metabolites of *E. lata* could not be detected in naturally infected vines showing foliar symptoms of Eutypa dieback also indicates that these metabolites may not be suitable chemical markers. Reasons for this remain unclear; it is possible that, following their entry into grapevine tissue, such metabolites are rapidly broken down into compounds that cannot be detected using HPLC. Alternatively, the suggestion of Mahoney et al. (2005) that such metabolites are not, in fact, translocated from the site of infection may also explain our inability to detect these compounds in the sap or foliage. Octave et al. (2006) isolated polypeptide compounds from a single isolate of *E. lata* grown in artificial culture and showed that when leaves were treated with such compounds, mesophyll cells underwent modifications similar to those observed in naturally infected vines. Hence, it is possible that such compounds may prove a more reliable indicator of infection by *E. lata*. However, before such research is initiated, it would be necessary to confirm that these polypeptides are produced by the majority of isolates of *E. lata*, and that they are not an artefact of the medium upon which the fungus is grown.

Acknowledgements

This project was supported by the Commonwealth Cooperative Research Centre Program and conducted by the CRC for Viticulture with support from Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funds from the Federal Government. Research conducted at the Western Regional Research Center was supported by a grant from the American Vineyard Foundation (Project V200). We thank M. Carter, M. Cole, M. Creaser, J. Péros and E. & J. Gallo Winery for providing isolates of fungi, and L. Hooper for technical assistance. We also thank Brian Loveys and Chris Soar (CSIRO Plant Industry) for assistance in using the pressure chamber, as well as Paul Kriedemann (ANU Research School of Biological Sciences) and two anonymous reviewers, for helpful comments on our manuscript.

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Manuscript received: 1 January 2006
Revised manuscript received: 12 May 2006